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# TEM as an Important Tool to Study Aquatic Microorganisms and their Relationships with Ecological Processes

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Thiago P. Silva, Juliana P. Gamalier and Rossana C.N. Melo

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## Abstract

Microorganisms are critically important for ecological processes in aquatic environments. Bacteria and viruses are key components of the microbial loop and are central for biogeochemical cycles in aquatic ecosystems. Our group has been using transmission electron microscopy (TEM) to study aquatic microorganisms in both natural tropical ecosystems and cultures. In this review, we highlight structural aspects of freshwater bacteria, based on TEM findings that have provided insights into the functional capabilities of these cells in aquatic tropical ecosystems. First, we focus on TEM applied to the study of the ultrastructural diversity and morphological alterations of bacteria in response to environmental stress. Second, we address the relationship between viruses and bacteria in freshwater ecosystems. Third, we demonstrate by TEM that outer membrane vesicles (OMVs), structures associated with cell secretion and cell communication, are released by aquatic bacteria into natural ecosystems and cultures. Thus, TEM has proven to be a powerful technique to study aquatic microorganisms, contributing to the understanding of ecological processes, including regulation of bacterial populations, during different environmental conditions.

**Keywords:** Transmission electron microscopy, freshwater bacteria, ultrastructure, aquatic ecosystems, cell viability, cell death

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## 1. Introduction

Aquatic microorganisms such as bacteria and viruses are critically important for ecological processes, for example, carbon cycling and energy flow in aquatic environments [1]. Bacteria are key components of the microbial loop in aquatic ecosystems, an alternative route of dissolved organic matter and nutrient transfer to metazoan trophic levels and consequently

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influence the flow of carbon and energy within an ecosystem [2, 3]. Viruses are remarkably abundant in aquatic ecosystems and within bacteria play an important role in the aquatic microbial loop. Viruses can infect bacteria and act in their mortality, thus exerting a significant control over aquatic bacterial and phytoplankton communities. Therefore, viruses can impact the pathways of matter and energy transfer in aquatic ecosystems [4, 5].

The understanding of the functional capabilities of microorganisms in microbial food webs and human health issues is largely dependent on methods applied to the direct visualization of them during physiological and environmental stress conditions. For example, individual imaging of bacteria is valuable to recognize bacterial viability and their physiological functions at single-cell level [6]. Our group has been using transmission electron microscopy (TEM) to study aquatic microorganisms, especially bacteria, from tropical ecosystems. The structural organization of these organisms has been investigated in water samples directly collected from natural environmental sites or kept in cultures. In this review, we highlight the ultrastructural aspects of freshwater bacteria, based on TEM findings, which have provided insights into the functional capabilities of these cells in aquatic tropical ecosystems.

## **2. Ultrastructure of freshwater bacteria: diversity and morphological alterations in response to environmental stress**

While observation of aquatic bacteria by light microscopy is an approach extensively used in studies of planktonic bacteria, the ultrastructure of these organisms is not completely understood [7-9]. In the past, bacteria were considered as prokaryotic microorganisms with a very simple ultrastructure. However, improvement of electron microscopy techniques and more refined analyses have revealed well-defined structures and higher levels of cell organization in bacteria [9].

In aquatic ecosystems, short-time physicochemical variations are frequent and affect environmental properties. Thus, bacterial communities need to be able to respond efficiently to fluctuating conditions of the aquatic environment [5, 10, 11]. On the other hand, bacterial cells can exhibit morphological and ultrastructural changes in response to environmental stress.

### **2.1. Ultrastructural diversity of bacteria from aquatic ecosystems**

The morphological diversity of bacteria goes far beyond a simple description of the bacterial shape, as frequently reported by ecological studies [12-16]. Bacteria from aquatic ecosystems have a complex cell ultrastructure with a cell envelope enclosing a cytoplasm with a variety of cell structures and compartments that can serve as organelles [17-19]. Freshwater bacteria, in addition of showing typical structures in the cytoplasm, such as nucleoid, granules, and lipid bodies, can exhibit intracellular membrane systems represented by mesosomes and thylakoid membranes. External structures such as cell envelope with distinct compositions, S-layer, external capsule, and extracellular vesicles, are also found in freshwater bacteria. The main bacterial structures depicted by TEM are listed in Table 1.

Structure	Morphological Description	Functions	Figures
<b>Cell envelope</b>	A complex multilayered structure which envelopes bacteria. Basically, there are two types of bacterial cell envelopes: (1) Gram-positive, composed of plasma membrane and cell wall and (2) Gram-negative, composed of plasma membrane, periplasm and outer membrane. Capsular structures and S-layers may also constitute the cellular envelope.	This structure serves to protect bacteria from their unpredictable and often hostile environment.	1A, 1Ai, 1B, 1Bi, 2A, 2Ai
<b>Plasma membrane</b>	A bilayer membrane seen under TEM as a classical trilaminar structure limiting the cell contents.	Plasma membrane acts as a permeability barrier for most molecules and serves as sites for transport of molecules into the cell. In addition, it is functionally associated with energy conservation as the location in which a proton motive force is generated.	1A, 1Ai, 1B, 1Bi, 2A, 2Ai
<b>Cell Wall</b>	Structural layer adjacent to the plasma membrane that appears as an electron-dense layer composed by peptidoglycans (gram-positive envelope) or a complex formed by periplasm and outer membrane (gram-negative envelope).	Cell wall provides structural integrity to the cell and prevents osmotic lysis.	1A, 1Ai, 1B, 1Bi, 2A, 2Ai
<b>Periplasm (periplasmic space)</b>	The periplasm is a concentrated gel-like matrix in the space between the inner plasma membrane and the bacterial outer membrane in gram-negative bacteria. This space is called periplasmic space. Gram-positive bacteria present this structure as a conspicuous space between the plasma membrane and the cell wall. Periplasm is filled with water and proteins and is therefore somewhat reminiscent of the cytoplasm	Periplasmic proteins have various functions in cellular processes including cell transport, cell degradation and cell motility.	1A, 1Ai, 1B, 1Bi, 2A, 2Ai
<b>S-Layer</b>	Cell surface protein layer that is composed of a two-dimensional array of proteins with a crystalline appearance.	Uncertain functions. It has been suggested that this layer acts as a partial permeability barrier for large substrates and provides resistance, adhesion and stabilization to the cell.	2A, 2Ai

Structure	Morphological Description	Functions	Figures
<b>Capsule</b>	Electron-lucent extracellular layer attached to the cellular envelope. This layer is formed by an exopolymeric matrix of polysaccharides.	Bacterial capsule has important functions related to cell recognition, defense and virulence.	2B, 2Bi
<b>Outer Membrane</b>	Outer bilayer membrane with a typical trilaminar appearance, delimiting gram-negative bacteria, and, for this reason, is a distinguishing feature of these bacteria. It is adjacent to the periplasmic space. The outer membrane composition differs from that of the inner plasma membrane, being composed of glycolipids, mainly lipopolysaccharides, which are located at its outer leaflet.	This membrane acts as a permeability barrier despite containing many passive transport channels. In addition, contributes for the increase of bacterial virulence.	3A
<b>Outer Membrane vesicles (OMVs)</b>	Spherical or rod-shaped vesicles, which are released from the outer membrane. OMVs are delimited by a bilayer membrane with typical trilaminar aspect and variable electron-density. OMVs can vary in size from 20 to 300 nm in diameter.	OMVs may contain proteins and other molecules that are related with cellular communication, defense, biofilm formation and DNA transfer.	3A, 3B, 3Bi
<b>Mesosomes</b>	Folded invaginations of the plasma membrane, which appear as tubular, vesicular or lamellar sacs.	Uncertain functions. It seems associated with cell division.	1A
<b>Thylakoid membranes</b>	System of lamellar membranes located in a large area of the cell cytoplasm.	These membranes serve as sites for the photosynthetic apparatus, enzymatic systems and electron transfer chains.	3A
<b>Granules</b>	Appear as spherical electron-dense structures in the bacterial cytoplasm.	These structures store a variety of organic and inorganic compounds.	1B, 2B, 2Bi
<b>Nucleoid</b>	Non-delimited electron-lucent areas in the cytoplasm. It is composed of DNA with a small amount of RNA and proteins.	Regulator center of cellular activities and cell replication.	1A, 1B, 2B, 2Bi
<b>Gas Vesicles</b>	Cylindrical tubes closed by conical end caps with perimeter size varying from 45 to 200 nm. They are mostly restricted to planktonic microorganisms (cyanobacteria and some bacterial species).	Gas vesicles promote cell buoyance in aquatic environments and enable vertical migration of cyanobacteria.	-

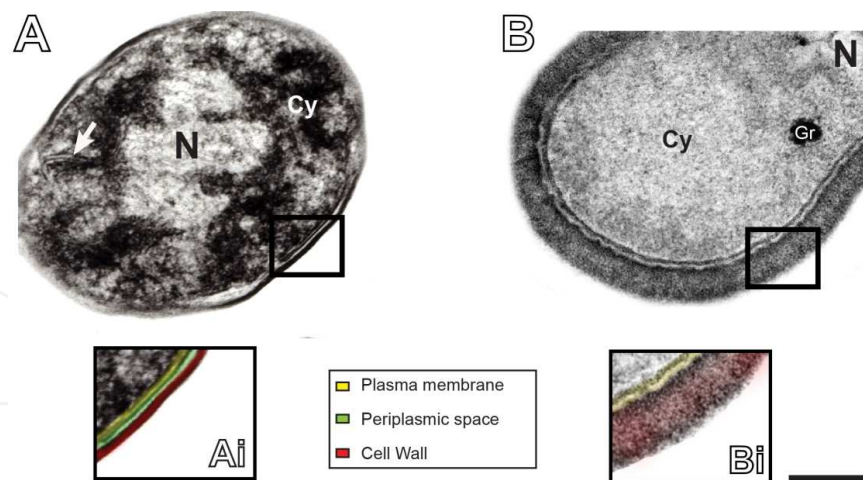
Structure	Morphological Description	Functions	Figures
<b>Lipid Bodies</b>	Electron-dense or electron-lucent spherical organelles surrounded by a half-unit membrane.	Lipid bodies store lipophilic compounds that are used as metabolic energy. However, they might be related with other - more complex yet unclear functions in prokaryotes and may have associated proteins.	
<b>Flagella</b>	Tubular filamentous structures attached to the cell surface. It is better observed by TEM when samples are negatively stained.	The flagellar filament is rotated by a motor apparatus in the plasma membrane and allows the motility of the cell in aquatic environments.	

**Table 1.** Main ultrastructural components of freshwater bacteria

TEM has been helping to understand the ultrastructural diversity among bacteria from aquatic ecosystems, associated with the presence of different internal and external structures. Our studies from tropical aquatic ecosystems have shown that ultrastructural diversity is an important aspect to be considered for better understanding of the role of these microorganisms. For example, variations in the cytoplasmic electron density (Figures 1 and 2) are frequently observed in freshwater bacteria and might reflect different stages of metabolism and/or differential molecular compositions. We also found a substantial variation in the bacterial cell envelope thicknesses (Figure 1) and compositions (Figures 1 and 2A), which are related to the presence of gram-positive and gram-negative bacteria, both commonly found in aquatic ecosystems. Our quantitative TEM analyses revealed a significant proportion of bacteria with a limiting capsule (Figure 2B and 2Bi). Our data showed that 31 % of freshwater bacteria had capsular structures [20]. This frequency is higher than that found in marine bacteria (7–27 %) [21–23]. This structural component is important for multiple functions, such as cell interaction with the environment, absorption and storage of nutrients, barrier against toxic agents from the medium and predation, and protection from viral infection and biofilm formation. Moreover, some bacteria showed particles adhered to the bacterial capsular structure (Figure 2v and 2Bi), which may be indicative of a survival strategy important for acquisition of organic or inorganic nutrients and protection against predators [24]. The well-defined coating formed by particles around bacteria, revealed by TEM observations, may act as an important micro-environment that is not identified by other techniques and open new frontiers in the understanding of bacterial ecology [25].

An interesting ultrastructural observation is the presence of membranous secretory vesicles projecting from the bacterium outer membrane into the extracellular medium in samples from





**Figure 1.** Ultrastructural views of aquatic bacteria collected from an Amazonian ecosystem. (A and B) In the cytoplasm (Cy), observe typical compartments and structures, as nucleoid (N), mesosomes (arrow), and granule (Gr). In (Ai) and (Bi), note the cell envelopes with different thicknesses and composed of plasma membrane (highlighted in yellow) and cell wall (red), with (Ai) and without (Bi) periplasmic space (green). Bacteria also show the cytoplasm with distinct electron-density. Reprinted from ref. [20] with permission. Scale bar: 160nm (A), 60nm (B), 120nm (Ai), and 30nm (Bi).

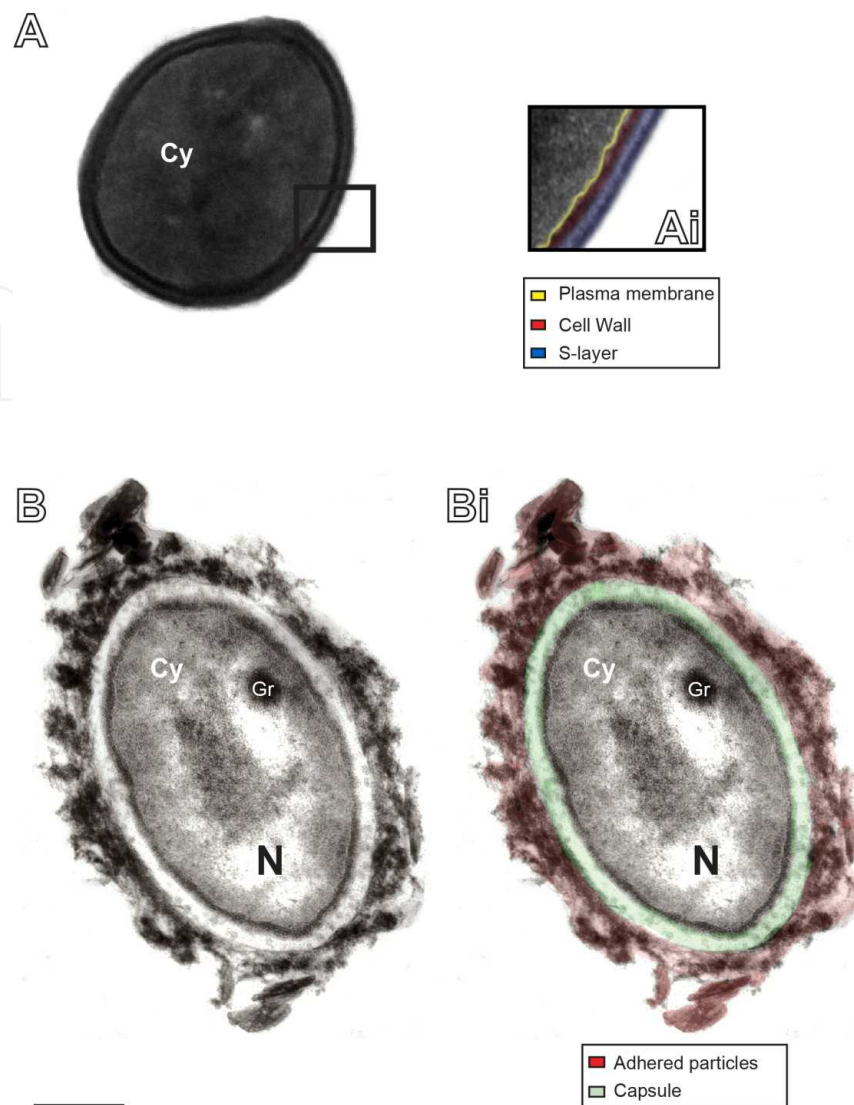
both natural environments and cultures (Figures 3A and 3B). This particular aspect is discussed in more detail in Section 3.0.

TEM also revealed that bacteria from aquatic ecosystems may exhibit a consistent system of endomembranes, — mesosomes and/or thylakoid membranes in the bacterial cytoplasm. Mesosomes (Figure 1A, arrow), considered as artifacts in the past, have, more recently, been receiving increasing attention because of their association with some cell functions, such as chromosome segregation during cell division. Intriguingly, mesosomes have been documented in bacteria in response to stress conditions [26]. The presence of thylakoids is a distinct morphological feature, found in cyanobacteria and a small group of bacteria [27] (Figure 3B). These endomembranes have a crucial function related to metabolic processes, particularly photosynthesis. Because thylakoids are unambiguously identified in high resolution by TEM, this technique is a reliable tool to distinguish between heterotrophic and autotrophic aquatic prokaryotes in environmental samples. Routine evaluation of these types of organisms currently relies on the use of light microscopy and appropriate fluorochromes, which do not enable detailed visualization of the thylakoids.

Our TEM data reinforce the fact that bacteria constitute structurally complex organisms and denote the functional complexity of these microorganisms, likely related to their metabolic and adaptive diversity [2, 24].

## 2.2. Ultrastructural alterations and death of bacteria in response to environmental stress

The physiological state of bacteria is an important parameter in aquatic ecosystems to understand variations on microbial communities and their potential impact on the food web and fluctuations in geochemical cycles in which these microorganisms are involved. Bacterial communities from aquatic ecosystems cannot be restrictively categorized as active or inactive,

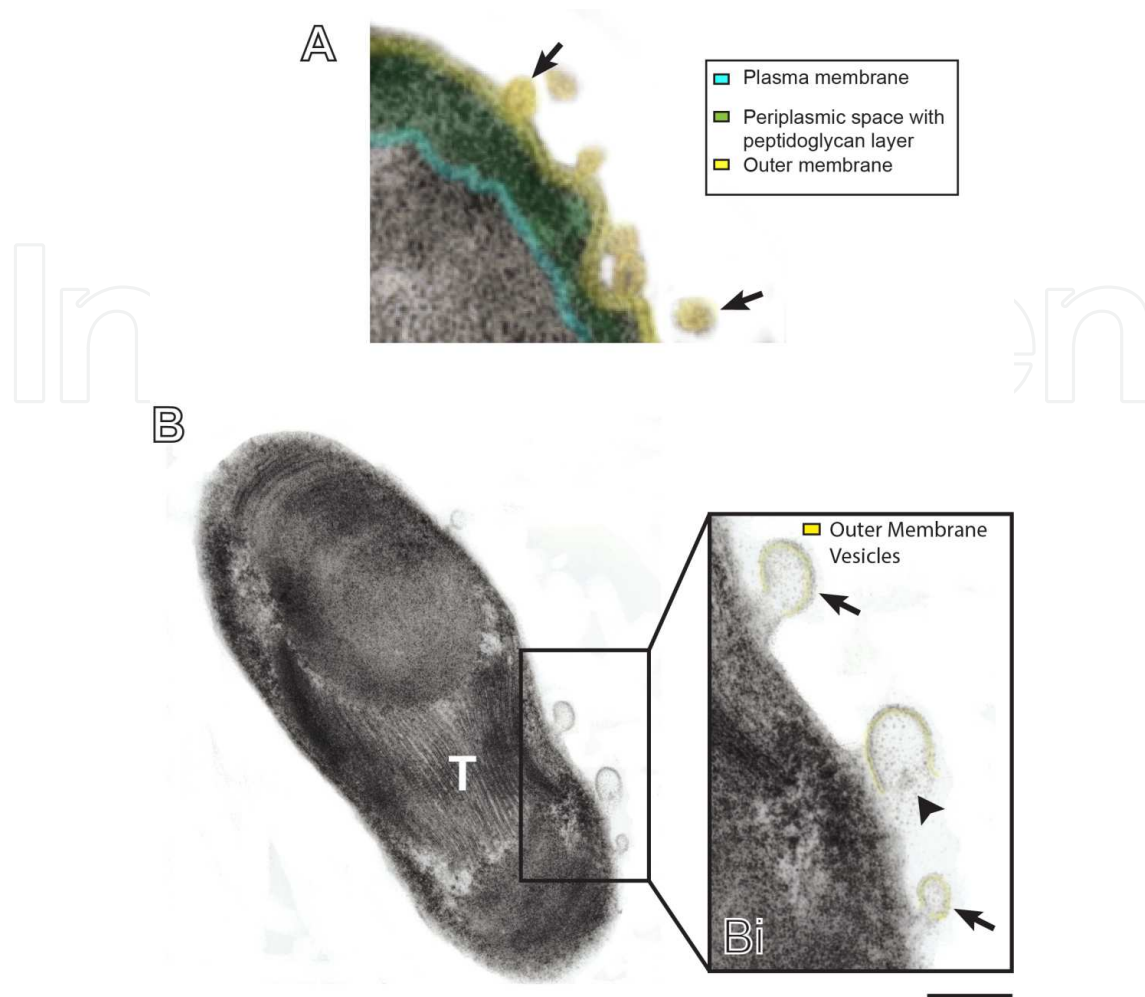


**Figure 2. Ultrastructural components of freshwater bacteria.** (A and Ai) A cultured bacterium shows the cell envelope composed by plasma membrane (highlighted in yellow), cell wall (red), and S-layer (purple). (B and Bi) Substratum particles (highlighted in red in Bi) are seen as an adhered coating localized externally to the capsular structure (highlighted in green in Bi) of a bacterium collected from a natural environment. Typical bacterial structures such as nucleoid (N) and granule (Gr) are observed in the cytoplasm (Cy). Figure 2B was reprinted from ref. [20] with permission. Scale bar: 130 nm.

since these cells present a continuous variation of their physiological state. From an ecological point of view, bacteria can be distinguished within microbial communities as viable/live cells, which play a functional role and participate in the production of biomass or dead cells, which no longer play a role in secondary production [28]. It is well documented a continuum of physiological status of bacterial life and death in aquatic ecosystems and a great variation in bacterial viability depending on factors such as heterogeneity of bacterial populations, environmental stress, nutrient competition and predation [6, 28-30].

Live/dead bacteria can be characterized by: (i) presence/absence of structures, (ii) genetic parameters, (iii) metabolism or functional activity, and (iv) reproduction and growth viability





**Figure 3. Ultrastructure of gram-negative bacteria from aquatic ecosystems.** Observe in (A), a typical gram-negative envelope of a bacterium exhibiting plasma membrane (highlighted in blue), periplasmic space with periplasmic layer (green) and outer membrane (yellow). In (B), an autotrophic aquatic bacterium shows thylakoids membranes (T), organized as a system of membranes in the bacterium cytoplasm. Note in (A) and (Bi) the formation of secretory vesicles from the bacterial outer membrane (arrows) and the release of vesicle contents into the extracellular environment (arrowhead in Bi). The trilaminar aspect of the outer membrane (highlighted in yellow) is clearly observed in high magnification in (Bi). Figure 3B was reprinted from ref. [20] with permission. Scale bar: 80 nm (A, Bi), 200 nm (B).

[31]. Yet, under an ecological perspective, the definition of bacterial life/death in aquatic ecosystems relies mostly on cell viability and growth analyses [32-36].

Although epifluorescence microscopy became the standard method for evaluating environmental bacteria death through indirect quantification of bacterial concentration (35-37), this approach, which is based on the use of routine fluorochromes such as DAPI and Acradine Orange, do not enable accurate assessment of the viability state of bacterial cells and may highlight other particles that are not necessarily bacteria (38). Moreover, this technique does not consider physiological aspects of bacterial cells [37-39]. More recently, other bacterial counting methods, which use more specific fluorescent dyes that consider the physiological aspects of bacterial cells, have been described [40]. However, TEM is the only technique with sufficient resolution to reveal morphological aspects indicative of cell viability and physiology,

enabling the detection of cell alterations that occur even before cell lysis. Therefore, bacteria with intact structures and bacteria presenting damaged cellular structures can be considered live or in process of death, respectively.

By studying impacted freshwater ecosystems in Brazil: Batata Lake (Amazonian region) that received tremendous amounts of bauxite tailings from a mining operation [41], and Funil Reservoir (Rio de Janeiro state) that received industrial, domestic, and erosive process effluents [42], we found several ultrastructural aspects indicative of bacterial cell death. The most frequent bacterial changes in response to environmental stress were: clumped granules (Figure 4A), cytoplasmic condensation (Figure 4B), structural damage of the cell envelope (Figure 4B), loss of cell shape (Figure 4C), and cell elongation (Figure 4D). Bacteria lacking internal structures known as “ghost bacteria” [43] were also observed (Figure 4A). Therefore, ultrastructural analyses were revealing in clarifying the effects of environmental stress on bacterial cell structures and bacterial dynamics in aquatic ecosystems.

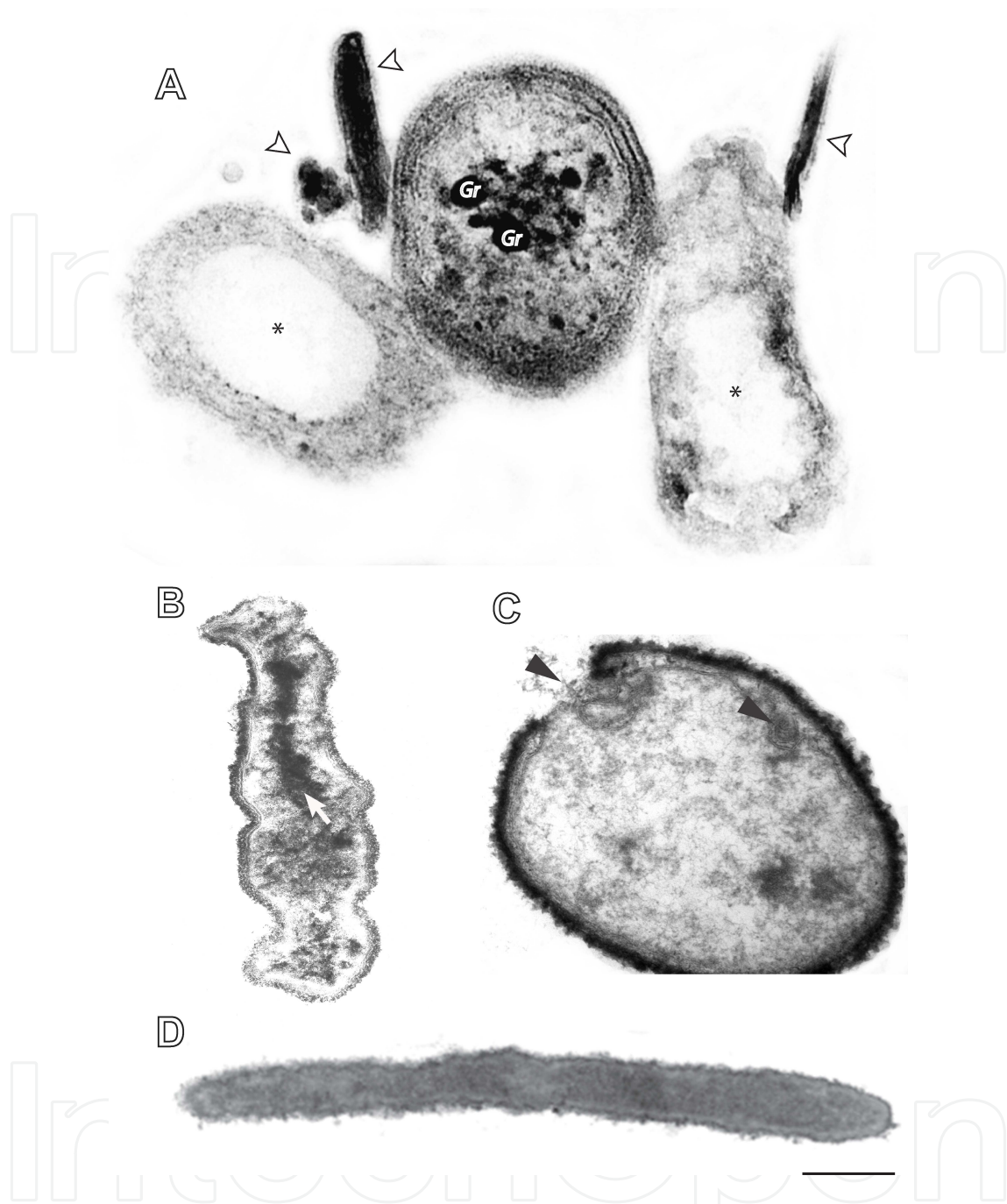
### 2.3. Visualizing virus-infected bacteria in aquatic ecosystems

Viruses are the smallest biological entities known. They are intracellular parasites, which can infect prokaryotic or eukaryotic cells. Viruses are ubiquitous in aquatic ecosystems, and increasing attention has been paid on their role in aquatic food webs since it was discovered that they are the most abundant aquatic components. Because viruses play an important biogeochemical function by releasing dissolved organic matter and nutrients through host cell lysis, they can affect various ecological factors, such as ecosystem respiration, primary production, genetic transfer between microorganisms, and species distribution [5].

TEM studies frequently report the occurrence of viral particles infecting bacteria termed bacteriophages [4, 29, 44]. Viruses are seen by TEM as small electron-dense particles with varied shapes and perimeter size varying from 20–200 nm (Figure 5). Viruses consist of genetic material (DNA or RNA, single- or double-stranded) surrounded by a protein coat (some also have lipids) [45]. They act on the control of bacterial population and are responsible for 40% of bacterial mortality in aquatic ecosystems [4, 46].

Bacteriophages have basically two different life cycles considering the onset of a viral infection until lysis of host cell: (1) Lytic cycle: viruses attach to host bacteria and inject their genetic material (DNA or RNA) into the cell, then they drive the host to produce numerous progeny viruses leading to bacterial cell burst and infection spreading to other cells. (2) Lysogenic cycle: viral genome integrates the genome of host bacteria and reproduces as genetic material without cell lyses. In this case, stress to the host bacteria can trigger a switch to lytic infection (lysogenic ↔ lytic) [5].

Our group has been investigating the relationship of virus—bacteria by TEM and has demonstrated an important correlation among free-living bacteria and virus in an Amazonian ecosystem (Batata Lake) [44]. Although there is a growing body of research on aquatic viral ecology, little is known about viral function in tropical ecosystems, particularly in Amazon environments [44, 47]. TEM revealed the occurrence of viruses with nearly spherical heads and without tails (Figure 5). The structure of the virus capsid with its repetitive morphological



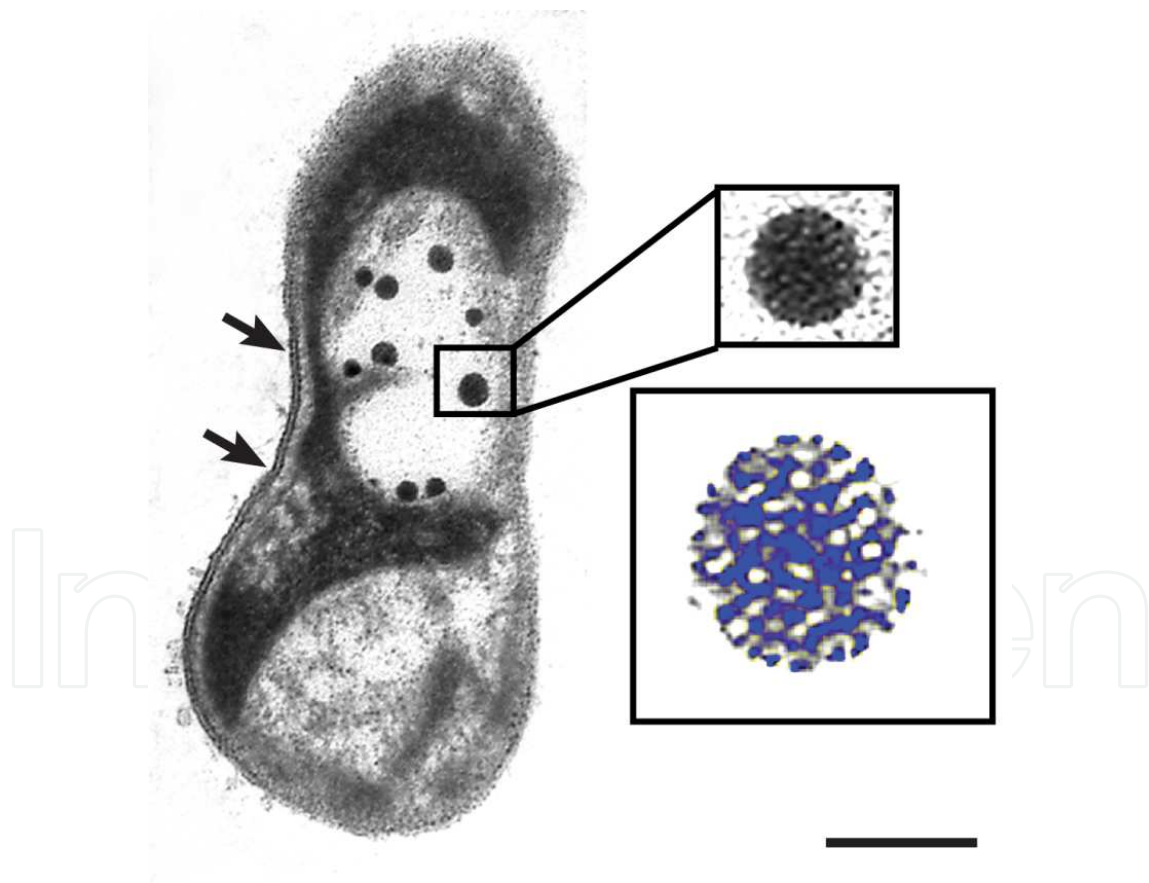
**Figure 4. Bacteria from impacted aquatic environments show clear ultrastructural alterations.** In (A), a damaged bacterium with clumped granules (Gr) is seen between two “ghost” bacteria, characterized by the presence of an empty cytoplasm (\*). Bacteria-associated cellular debris is observed (A, white arrowhead). In (B), note bacterial cytoplasmic condensation (arrow) and loss of cell shape while in (C) a clear structural damage of the cell envelope is observed (arrowheads). Cell elongation is shown in (D). Water samples were collected from Batata Lake (Amazonian region, Brazil), immediately fixed and processed for TEM as ref. [20]. Scale bar: 180 nm (A, B, and C) and 350 nm (D).

units occasionally could be observed in some cells (Figure 5, boxed area). On the other hand, some infected bacteria lacked an intact cell membrane or were partially empty. This morphological aspect indicates that viruses can induce bacterial cell death, which is associated with the lytic cycle of the virus in aquatic environments.

Our data demonstrated that a variable number of phages are present within virus-infected bacteria. TEM quantitative analyses showed that 34.2% of bacteria had viruses in the cytoplasm (Figure 5), with  $10.0 \pm 3.5$  (mean  $\pm$  SEM) phages per cell-section. Additionally, we have found virus-infected bacteria in cultured samples from Funil Reservoir, indicating that the presence of viruses in tropical ecosystems is a broad event.

Several environmental factors, including solar radiation and temperature, can influence viral abundance. Exposure to UV radiation decreases viral abundance, while low temperatures decrease their capability of infection in aquatic ecosystems [48-50]. It is also described that the increase of organic matter and anthropogenic pollutants increase the abundance of viral particles in water environments [44, 47, 51].

Altogether, our ultrastructural data showed a variable number of viruses within the bacterial cytoplasm, which demonstrates a clear interaction between these organisms. Assessment of viral production and virally caused mortality of bacteria are crucial parameters to understand the detailed role of viruses in food webs.



**Figure 5. A virus-infected bacterium from an aquatic ecosystem shows several phages.** The boxed area shows the virus capsid structure at high magnification. Note that the capsid is composed of repetitive morphological units (highlighted in blue at a higher magnification). The trilaminar structure of the plasma membrane is partially observed (arrows). Scale bar: 266 nm, 80 nm (Box, virus at high magnification), and 40 nm (Box, highlighted in blue). Reprinted from ref. [44] with permission.



### 3. Production of outer membrane vesicles by freshwater bacteria

In recent years, the extracellular release of membrane-bound vesicles by prokaryotic cells has become the subject of great interest. In prokaryotes, these vesicles are frequently extruded from the outer membrane (OM) of gram-negative bacteria and cyanobacteria, and, for this reason, they are known as outer membrane vesicles (OMVs). By TEM, OMVs appear as spherical or rod-shaped vesicles enveloped by a double membrane with variable electron-density content and diameter size varying from 20 to 300 nm [52-54] (Figure 3).

OMVs have been shown to contribute to diverse bacterial processes, such as pathogenesis [55, 56], cellular defense [53, 57], cell-to-cell communication [58], and DNA transfer [52, 59]. OMVs are able to store and transport a broad range of cargo repertoire from bacterial periplasm and cytoplasm, that can explain the variable electron-density observed by TEM. Thus vesicular transport represents a relevant signal trafficking system in prokaryotes (reviewed in [54, 60]). Despite the numerous ways in which vesicles may affect microbial communities, their abundance and potential functions in aquatic ecosystems remain unknown. Recently, these vesicles were recognized as abundant and important to carbon flux in marine ecosystems [61]. Vesicle release occurs during the normal growth of many species, and although growth conditions, stressors, and membrane structure can influence the number of vesicles produced, the regulation of vesicle production is still unclear.

By studying microorganisms from freshwater ecosystems in both natural environmental and cultures through TEM, we have identified a consistent production of OMVs by bacteria [20]. These vesicles were round, delimited by classical membrane with trilaminar appearance, and exhibited morphology similar to those described on the surfaces of other bacterial species [52] (Figure 3). They appeared attached to the outer membrane of the bacteria with typical gram-negative envelope or free in the extracellular environment (Figure 3).

Our data from samples collected from Batata Lake suggest that OMV-mediated secretion is an important cell process of freshwater bacteria (Figure 3). Although the function of OMV remains to be defined, these secretory vesicles, observed for the first time by us in aquatic bacteria from a tropical ecosystem [20], may be important for bacterial survival and inhibition of lysis induced by viral infection that is relevant in this ecosystem [44], as mentioned before. Moreover, OMVs may be relevant in the transport of products involved in the formation of the cell wall, inhibition of toxic components present in the surrounding environment, and formation of biofilms. They may also be associated with delivery of enzymes for nutrients acquisition and autolysins for degradation of other bacteria favoring the competition for niches. All these environmental factors may be more prominent in an impacted ecosystem.

### 4. Concluding remarks and perspectives

Although our understanding of the biological aspects of bacteria from aquatic ecosystems has advanced significantly, our knowledge of the structural organization of these ecologically

important microorganisms is still incomplete. It is unknown how bacteria differ in their cellular architecture and respond at the structural level to abiotic and biotic stress in aquatic environments. This knowledge is essential for an integrative understanding of the bacterial physiology and ecology. TEM has helped to elucidate the internal organization of aquatic bacteria at the nanometer scale. Earlier views of the ultrastructure of these microorganisms, considered in the past as cells with a very simple structure, are now being expanded to encompass a new understanding of their multifunctional activities and cellular complexity. Our results from environmental and culture-based TEM studies have revealed an ultrastructurally diverse population of bacteria in freshwater ecosystems, characterized by distinct cytoplasmic and external structures. The recognition that these microorganisms have cytoplasmic membranes and are able to release membrane-bound vesicles may be crucial to the understanding of their functional capabilities. Several aspects of the bacteria life remain to be defined. For example, it is not understood how bacteria interact with each other in aquatic ecosystems. Is there a regulated vesicular transport-mediated secretion from/to bacterium? If yes, can this pathway be blocked or stimulated by a cell stressor? These and other aspects, including the bacterial responses to several environmental stresses, mechanisms of bacterial cell death and the bacteria–viruses interaction, need to be investigated in more detail so that the functional significance of bacteria and other microorganisms from aquatic ecosystems can be fully appreciated as critical regulators of ecological processes.

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## Author details

Thiago P. Silva, Juliana P. Gamalier and Rossana C.N. Melo\*

\*Address all correspondence to: [rossana.melo@ufjf.edu.br](mailto:rossana.melo@ufjf.edu.br)

Laboratory of Cellular Biology, Department of Biology, Federal University of Juiz de Fora (UFJF), Juiz de Fora, MG, Brazil

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